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Chloroplast DNA Phylogeography of *Pedicularis resupinata* (Scrophulariaceae) in Japan

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Chloroplast DNA variation in *Pedicularis resupinata* L. (Scrophulariaceae) was studied in 48 populations from the Japanese archipelago and adjacent regions. Nine distinct cpDNA haplotypes, based on the intergenic spacer between the *trnL* (UAA) 5'exon and *trnF* (GAA), were recognized. One cpDNA haplotype was distributed widely (type A), and most of the other haplotypes were found to be geographically structured. Two major clades (I and II) were revealed in phylogenetic analyses among the haplotypes by adding the sequence data from the intergenic spacers of *trnT* (UGU) and *trnL* (UAA) 5' exon, and *atpB* and *rbcL*. The haplotypes of Clade I were widely distributed not only within the Japanese archipelago, but also on the Korean Peninsula and Sakhalin. Clade II, however, was found only in plants from central Honshu and Shikoku, Japan. These results suggest that the cpDNA haplotypes of Clade II originated within the Japan and remain as relics in central Honshu and Shikoku. The Japanese origins of two endemic taxa, *P. resupinata* var. *caespitosa* Koidz. and var. *microphylla* Honda are also discussed.

Key words: chloroplast DNA, endemic taxa, haplotypes, Japanese archipelago, origin, *Pedicularis resupinata*, Scrophulariaceae

It has been generally considered that the Japanese flora was derived by invasions of plants from the Eurasian or Asian continents via Sakhalin, the Kuril islands, the Korean peninsula, and the Ryukyu islands throughout past geological epochs (Hotta 1974, Maekawa 1998). As evidences for this hypothesis are the many widespread species in the Japanese flora that are also extant on Asian continent. It also has been reported that about 40% of the plant species in Japan are endemic (Hotta 1974, Murata 1977), indicating that relatively many of the plants in Japan originated or differentiated within the Japanese archipelago. To understand the processes in the evolution of the Japanese flora, it is important to clarify the origin of Japanese endemic groups. There are few studies, however, of the phylogenetic reconstruction divergence patterns of such groups in the Japanese flora, except for studies of plants of the Ryukyu Islands (Hiramatsu *et al.* 2001, Setoguchi 2001) and on Japanese alpine plants (Fujii *et al.* 1997, 1999).

Pedicularis resupinata L. (Scrophulariaceae) is a perennial herb of sunny meadows from the lowlands to the subalpine zone in Japan. It is distributed in eastern Asia in Japan, Korea, northern to central China, Manchuria, Mongolia, Siberia, Sakhalin and Kamchatka (Yamazaki 1993). The species exhibits considerable diversity in external morphology and several infraspecific taxa have been proposed for plants in Japan. For example, Yamazaki (1981)

recognized four varieties: *resupinata*, *oppositifolia* Miq., *caespitosa* Koidz., and *microphylla* Honda. Yamazaki (1993) recognizes five varieties: *resupinata*, *oppositifolia* [not explicitly cited in Flora of Japan], *mikawana*, *teucriifolia* and *caespitosa*. Variety *microphylla* Honda is not mentioned and var. *resupinata*, although appearing in the key, is not included under the species description. Although the infraspecific treatment differs among taxonomists, varieties *caespitosa* and *microphylla* are accepted widely (Ohwi 1953, Kitamura *et al.* 1957, Yamazaki 1981). According to Yamazaki (1981), all four taxa are found in the Japanese archipelago. Varieties *caespitosa* and *microphylla* are endemic to Japan, implying that they originated there. Yamazaki (1981) inferred the evolutionary history of the group from observations of their external morphology. He hypothesized that (1) var. *resupinata* entered the Japanese islands from the north and that var. *caespitosa* became differentiated from var. *resupinata* in central Honshu; (2) plants of var. *oppositifolia* entered Japan by way of the Korean peninsula and var. *microphylla* became differentiated from var. *oppositifolia* in central Honshu. This hypothesis has not yet been verified using other evidence.

The chloroplast DNA (cpDNA) variation provides an opportunity for phylogeny reconstruction at the population level. The geographic structuring of variation may provide insights into the historical biogeography of species (Soltis *et al.* 1997, Soltis & Soltis 1998). Analysis of the relationships between gene phylogeny and geographic distribution of the phylogenetic groupings within a species is termed "intraspecific phylogeography" (Avise *et al.* 1987, Avise 2000). The chloroplast genome is an attractive target for such studies because it is effectively haploid, present in multiple copies and does not recombine (Hillis & Moritz 1990). In most angiosperms cpDNA is transmitted through the seeds and not through pollen, although in some plant species it is biparental or paternal (Mogensen 1996). CpDNA generally reflects seed flow and is therefore a good marker for monitoring colonization processes (McCauley 1994).

In this study, to elucidate the evolutionary patterns and processes of infraspecific diversification, I investigated the cpDNA variation and phylogeographic patterns in 48 populations of *Pedicularis resupinata* from the Japanese islands and adjacent regions. On the basis of the results, I discuss the origin of the Japanese endemics, vars. *caespitosa* and *microphylla*, and evaluate Yamazaki's hypothesis.

Materials and Methods

Sampling materials and total DNA extraction

I sampled a total of 172 plants from 48 populations of *Pedicularis resupinata* in the Japanese islands, the Korean peninsula, Sakhalin, and the Kuril islands (Fig. 1 and Table 1). The samples of *P. yezoensis* (sect. *Pedicularis*), *P. schistostegia* (sect. *Bicuspidatae*), and *P. chamissonis* (sect. *Orthosiphonia*) were also collected to use as outgroups of *P. resupinata* (sect. *Pedicularis*). Identification of the infraspecific taxa of *P. resupinata* is mainly according to Yamazaki (1981) (Table 1). I used the following morphological characteristics to identify the samples: inflorescence elongate or shortly spicate or capitate, leaves opposite or alternate, and leaf size. The leaves were dried and preserved in silica gel. Total genomic DNA was extracted from 0.01 g of dried leaves using the slightly modified CTAB method of Doyle & Dickson (1987).

Outline of methods for detecting cpDNA variation

For all plants, the non-coding region between the *trnL* (UAA) 5'exon and *trnF* (GAA) of cpDNA (Taberlet *et al.* 1991) was used for the recognition of cpDNA haplotypes by direct sequencing and/or by single-strand conformation polymorphism analysis of PCR-amplified fragments (PCR-SSCPs) methods (Fujii *et al.* 1997). The latter method was used

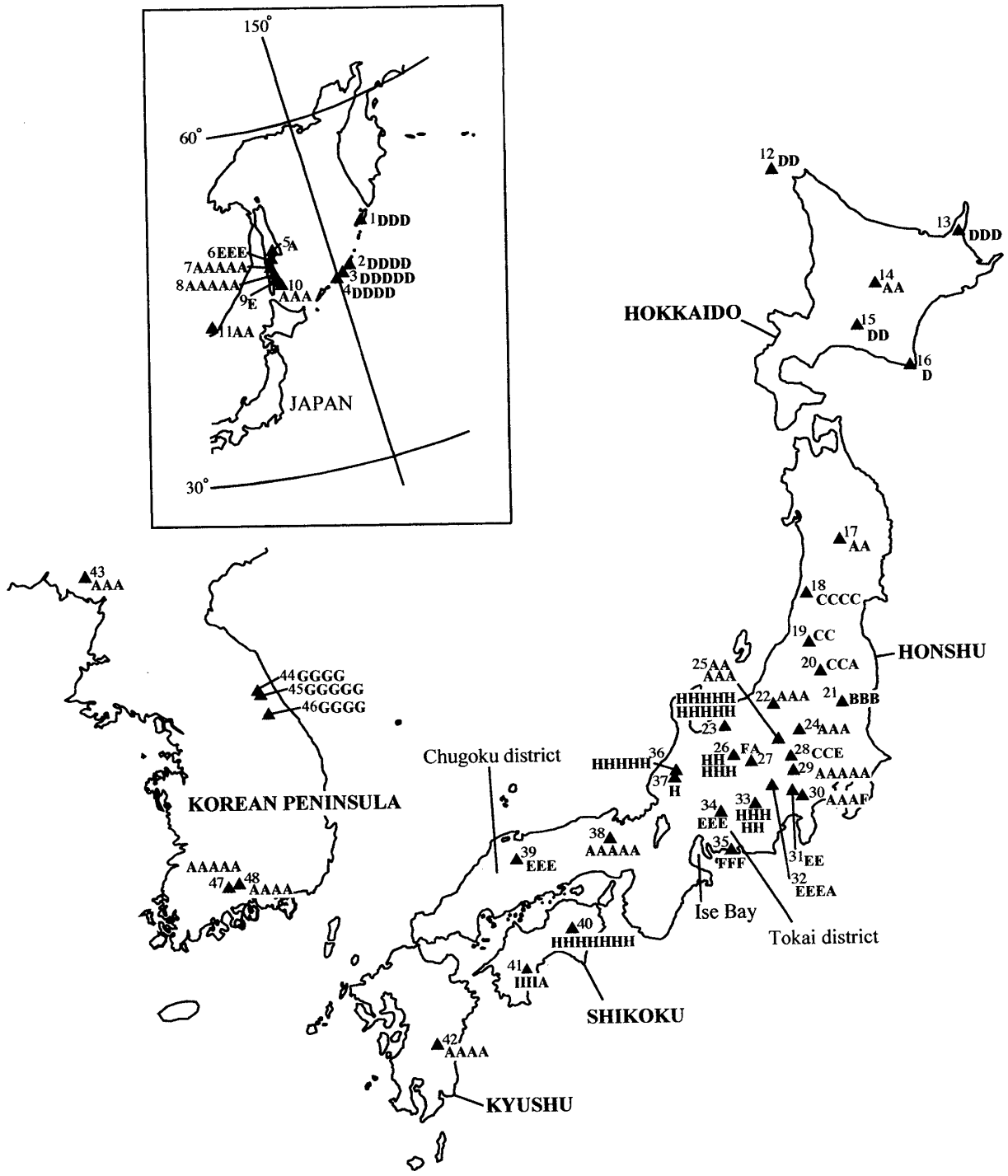

FIG. 1. Collecting sites of *Pedicularis resupinata* used in cpDNA analysis. Numbers in figure correspond to population numbers in Table 1. Letters represent cpDNA haplotype of individuals (see Table 2) based on non-coding region between *trnL* (UAA) 5'exon and *trnF* (GAA).

for the detection of intra-populational variation because it is a convenient and economical method. Thirty-five plants from 12 populations were analyzed by direct sequencing, 96 plants of 35 populations were analyzed by PCR-SSCPs methods, and 41 plants from 31 populations were analyzed by both methods. To infer the phylogenetic relationships among the cpDNA haplotypes detected, one sample representative of each haplotype (for a detailed description, see below) of two non-cod-

TABLE 1. Materials, and their sources, analyzed for cpDNA variation of *Pedicularis resupinata*.

Population No.	Locality and voucher[1]	No. of plants	Taxon[2]	CpDNA haplotypes[5]
Pedicularis resupinata L. (sect. *Pedicularis*)				
Russia				
1	Northern Kuriles: Onekotan Island, Alt. 100m, *H. Takahashi 28119* (SAP)	3	RES	D
2	Central Kuriles: Simushir Island, Alt. 20m, *H. Takahashi 28353, 67, 69* (SAP)	5	RES	D
3	Central Kuriles: Brat Chirpoev Island, Alt. 40m, *H. Takahashi 28466* (SAP)	4	RES	D
4	Central Kuriles: Urup Island, Alt. 10m, *H. Takahashi 28573* (SAP)	4	RES	D
5	Central Sakhalin: 25 km west of Poronaysk, *H. Takahashi 29621* (SAP)	1	RES	A
6	Southern Sakhalin: Pugachevskye (Maguntan), *H.Takahashi 27873* (SAP)	3	RES	E
7	Southern Sakhalin: 80 km north of Dolinsk, *H. Takahashi 29555* (SAP)	5	RES	A
8	Southern Sakhalin: 20km east of Sokol town, *H. Takahashi 29106, 22* (SAP)	5	RES	A
9	Southern Sakhalin: 20km southeast of Yuzhno-Sakhalinsk, *M. Suzuki 805021*(TUSG)	1	RES	E
10	Southern Sakhalin: 10 km east of Korsakov, *H. Takahashi 29421* (SAP)	3	RES	A
11	Primorskij (N44° 07' 18.3", E:135° 39' 24.2"), *C. Suyama 1754, 55* (KANA)	2	OPP	A
Japan				
12	Northern Hokkaido: Rebun Island, Alt. 70m, *KANA199010*	2	RES	D[6]
13	Eastern Hokkaido: Mt. Rausu, Rausu, Alt. 1300m, *KANA199005*	3	unknown[3]	D
14	Central Hokkaido: Daisetsu Lake, Alt. 1000m, *KANA199008*	2	OPP	A
15	Central Hokkaido: Mt. Yubari, Alt. 1300m, *KANA199009*	2	OPP	D
16	Southern Hokkaido: The Erimo Cape, Alt. 10m, *KANA199006*	1	OPP	D
17	Northern Honshu: Iwate, Mt. Yakeishi, Alt. 1300m, *KANA199012-13*	2	OPP	A
18	Northern Honshu: Yamagata, Mt. Gassan, Alt. 1500m, *MAK304317, 18*	4	OPP	C[6]
19	Northern Honshu: Yamagata, Mts. Iide, Alt. 2000m, *KANA199002-3*	2	OPP	C
20	Northern Honshu: Fukushima, Mt. Nanatsugadake, Alt. 1500m, *MAK302897, 98*	3	OPP	A and C
21	Northern Honshu: Fukushima, Mt. Kimen, Alt. 1400m, *MAK309687-89*	3	OPP	B[6]
22	Central Honshu, Gunma, Mt. Hakkensan, Alt. 1700m, *MAK304466, 68, 71*	3	OPP	A
23	Central Honshu, Nagano, Mt. Amakazari, Alt. 1800m,*MAK322468, 74, 75*	10	OPP	H
24	Central Honshu, Gunma, Mt. Jizou, Alt. 1600m, *MAK04324*	3	OPP	A
25	Central Honshu, Nagano, The Ikenotaira Marsh, Alt. 2000m, *MAK322471*	5	OPP	A[6]
26	Central Honshu, Nagano, Mt. Chogadake, Alt. 2500m, *MAK322472, 73*	5	CAE	H[6]
27	Central Honshu, Nagano, The Kirigamine Highlands, Alt. 1500m, *MAK307621, 23*	2	OPP	A and F
28	Central Honshu, Saitama, Mt. Futago, Alt. 1000m,*MAK303108-10*	3	OPP	C and E
29	Central Honshu, Yamanashi, The Ichinose Highlands, Alt. 1300m, *MAK310809-11*	5	OPP	A
30	Central Honshu, Yamanashi, Mt. Mikuni, Alt. 1200m, *MAK3041773*	4	OPP	A and F
31	Central Honshu, Yamanashi, The Suzuran Pass, Alt. 1400m, *MAK304325, 26*	2	OPP	E
32	Central Honshu, Yamanashi, Mt. Amari, Alt. 1600m, *MAK304464-67, 70*	4	OPP	A and E
33	Central Honshu, Nagano, The Sanpuku Pass, Alt. 2500m, *MAK322469-70*	5	CAE	H
34	Central Honshu, Gifu, The Hokonoko Lake, Alt. 800m, *MAK304320*	3	MIC	E[6]
35	Central Honshu, Aichi, Mt. Ishinomaki, Alt. 140m, *MAK303502*	3	MIC	F[6]
36	Central Honshu, Ishikawa, Mts. Hakusan (Ichinose), Alt. 1500-1700m	5	unknown[4]	H
37	Central Honshu, Ishikawa, Mts. Hakusan (Sannomine), Alt. 1800m, *MAK304323*	1	OPP	H
38	Western Honshu, Hyogo, Mt. Hyonosen, Alt. 750m, *MAK328503-08*	5	OPP	A
39	Western Honshu, Tottori, Mt. Daisen, Alt. 800m, *MAK304322*	3	OPP	E
40	Shikoku, Tokushima, Mt. Tsurugi, Alt. 1670m, *MAK316023, 322933-34*	7	OPP	H
41	Shikoku, Kochi, The Tengu Highlands, Alt. 1200-1300m, *MAK303237-41*	5	OPP	A and I[6]
42	Kyusyu, Kumamoto, Mt. Ichifusa, Alt. 1700m, *MAK303242-45*	4	OPP	A

TABLE 1. (continued)

Population No.	Locality and voucher[1]	No. of plants	Taxon[2]	CpDNA haplotypes[5]
North Korea				
43	P'yong-anbuk-do (N40° 56' 14.0", E124° 46' 53.2"), Coll. H. Kim, Alt. 600m	3	unknown[4]	A
South Korea				
44	Kang-won-do, Mts. Soraksan (Anasn), Alt. 900-1260m, *MAK322480-83*	4	OPP	G[6]
45	Kang-won-do, Mts. Soraksan (Mishiryong), Alt. 800-1260m, *MAK322485-88*	5	OPP	G
46	Kang-won-do, Mt. Odaesan, Alt. 800m, *MAK322494, 95*	4	OPP	G
47	Kyongsangnam-do, Mts. Chirisan (Nogodan), Alt. 880-1300m, *MAK322516-20*	5	OPP	A
48	Kyongsangnam-do, Mts. Chirisan (Chotdaebong), Alt. 1000-1400m, *MAK322500-03*	4	OPP	A
		Total 172 plants		
Outgroups				
Pedicularis yezoensis Maxim. (sect. *Pedicularis*)				
	Central Honshu: Nagano, Mt. Chogadake, Alt. 2500m, *MAK310467*			
P. schistostegia Vved. (sect. *Bicuspidatae* Steven)				
	Northern Hokkaido: Rebun Island, Alt. 20m, *KANA198102*			
P. chamissonis Steven (sect. *Orthosiphonia* H. L. Li)				
	Central Honshu, Nagano, Mt. Amakazari, Alt. 1900m, *MAK310461*			

[1] SAP: Hokkaido Univeristy, TUSG: Tohoku University, KANA: Kanazawa University, MAK: Makino Herbarium

[2] The intraspecific taxonomy of the P. resupinata were followed by Yamazaki (1981). The four abbreviations indicate the variety of the species as follows: RES; var. *resupinata*, OPP; var. *oppositifolia*, MIC; var. *microphylla*, CAE; var. *caespitosa*.

[3] The plants of the specimen were very young, and we could not identify them.

[4] No voucher specimen.

[5] The alphabetical characters indicate the cpDNA haplotypes observed in each population, see also Fig. 1.

[6] The samples were sequenced three cpDNA regions (*trnT-L*, *trnL-F*, and *atpB-rbcL*) for the present phylogenetic analyses.

ing regions between *trnT* (UGU) and *trnL* (UAA) 5'exon, and *atpB* and *rbcL* were further sequenced.

PCR amplification and sequencing

Three cpDNA regions were amplified by PCR for nucleotide sequence variation or for PCR-SSCPs as follows: the non-coding regions between the *trnT* (UGU) and *trnL* (UAA) 3'exon, the *trnL* (UAA) 5' exon and *trnF* (GAA) (Taberlet *et al.* 1991), and *atpB* and *rbcL* (Terachi 1993, Fujii *et al.* 1997). The PCR reaction mixtures contained 50-100 ng template DNA, 5μL of 10× PCR buffer (Takara, Japan), 0.2 mM of each deoxyribonucleotide, 2.0 mM of $MgCl_2$, 0.4μM of each of the primer pairs, and 1.0 U of ExTaq DNA polymerase (Takara) in a total volume of 50μL. The PCR program ran for 3 min at 94°C for initial denaturation, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min. The reactions were then extended by 7 min at 72°C.

The PCR products for direct sequencing were excised from 1% agarose gels and purified using a GENECLEAN II Kit (BIO 101, Inc.) to remove the non-incorporated primers and nucleotides. Sequencing reactions were carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc.). The sequencing reaction products were purified, concentrated by EtOH precipitation, and then applied to an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Inc.) with Long

Ranger gels (FMC BioProducts, USA).

Analysis of PCR-SSCPs

PCR-SSCPs of the non-coding region of *trnL* (UAA) 5'exon and *trnF* (GAA) of cpDNA were detected by following the procedure of Fujii *et al.* (1997). This region had long sequences (910-930 bp) and was digested by restriction enzymes (*Taq* I, Takara Co., Japan) to generate an adequate length of DNA fragments for SSCP. Gel composition for electrophoresis was 0.5×TBE (45 mM tris-borate, pH 8.3, 1 mM EDTA), 5% glycerol and 0.5×MDE gel solution (FMC BioProducts). Electrophoresis was performed in 0.5×TBE at 250 V for 4.5 h. The gel temperature was kept at 11.5 C by use of a thermostat-controlled water circulator.

Sequence alignment and phylogenetic analyses

Alignment of the sequences was done manually using the DNASIS-Mac program (Hitachi Software Engineering, Japan). Boundaries of the coding and non-coding regions were determined by comparing the sequences to the corresponding sequences in *Nicotiana tabacum* L. (Shinozaki *et al.* 1986). Insertion/deletions (indels) were generally placed so as to maximize the number of matching nucleotides in corresponding sequences. I determined the cpDNA haplotypes based on the site changes and indels of the non-coding region between *trnL* (UAA) 5'exon and *trnF* (GAA).

Phylogenetic relationships among the cpDNA haplotypes of *P. resupinata* were inferred by the maximum parsimony (MP) and neighbor-joining (NJ) methods, using three non-coding regions (*trnT-trnL*, *trnL-trnF*, and *atpB-rbcL*). The most parsimonious trees were obtained with the PAUP* 4.0 program (Swofford 2002) using the branch and bound search option. The relative levels of support for different clades was estimated using bootstrap analysis (Felsenstein 1985) based on 1000 replicates and decay analysis (Bremer 1988, Donoghue *et al.* 1992) using Autodecay version 4.0 programs (Eriksson *et al.* 1998). In the MP method, I used a data set including both substitution data and indel characters. Indels in the aligned sequences were coded as 1/0 binary characters in the data matrix. Gaps of more than 1 bp in length were treated as being due to single events. All character states, including indels, were specified as unordered and equally weighted. For the NJ tree, I calculated genetic distances, based solely on substitution data, using Kimura's (1980) two-parameter model in PAUP* 4.0. The bootstrap analysis was conducted with 1000 replicates.

Results

CpDNA haplotypes detected

To recognize the cpDNA haplotypes of *Pedicularis resupinata*, I determined the sequences of the non-coding region between *trnL* (UAA) 5'exon and *trnF* (GAA) of cpDNA. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank DNA databases under accession numbers AB110781, 110782, 110828, 110912 to 110930. The length of the region varied from 910-930 bp, and the region included 50 bp of the coding region of *trnL* (UAA) 5'exon and 32 bp of the coding region of *trnF* (GAA). The polymorphic characters in the region included 43 nucleotide substitutions and 17 indels among all accessions, and 12 site changes and eight gaps among the populations of *P. resupinata*. In the PCR-SSCP analysis, the *trnL-F* regions of most individuals were digested into two or three regions by *Taq* I, and I observed four, five, or six SSCP bands in each lane (Fig. 2). When a sample exhibited a band pattern of the same sequence as the known sample, I estimated that both samples had the same nucleotide sequence. Through the analysis, I was able to find intra-populational variation in cpDNA in six populations (No. 20, 27, 28, 30, 32, and 41, see Table 1).

Based on the 12 substitutions and four of the eight gaps detected, I was able to recognize nine dis-

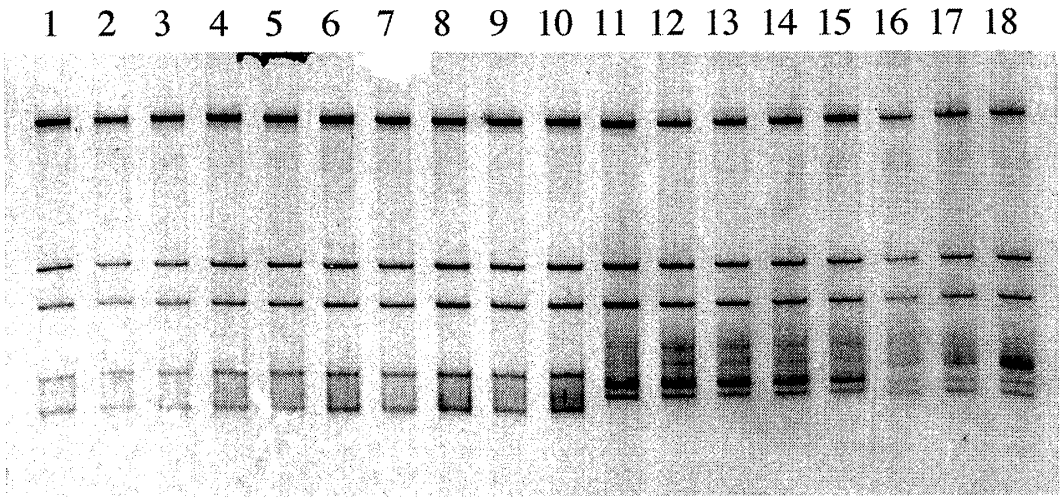

FIG. 2. Example of SSCP band patterns of cpDNA (non-coding region between *trnL* (UAA) 5'exon and *trnF* (GAA)) of *Pedicularis resupinata* from four populations: 1-5; Mt. Amakazari (population No. 23, see Table 1 or Fig. 1), 6-10; Mt. Chogadake (26), 11-15; Ichinose Highlands (29), 16-18; Ikenotaira Marsh (25). Intra-populational variation not shown.

tinct cpDNA haplotypes in *Pedicularis resupinata* (Types A-I; Table 2). For the purpose of identifying cpDNA haplotypes and performing phylogenetic analysis, four indels were omitted. These omissions were located at sequence positions 104-106, 142-146, 555-557 and 864-871 in this region (data not shown). These indels were mostly poly-A or poly-T variations, and the mutual homology of their sites was ambiguous. Haplotype A was found to be distributed widely throughout the Japanese islands, the Korean peninsula, northeastern Russia, and Sakhalin (Fig. 1). Most of the remaining haplotypes were geographically localized. Haplotype H was found in the highlands of central Honshu (23, 26, 33, 36, and 37) and on Mt. Tsurugi in Shikoku (40). I recognized haplotype C in four populations from northern Honshu (18, 19, 20, and 28), and haplotype F in the three populations from central Honshu (27, 30, 35). Most populations on the Kuril islands and in Hokkaido, Japan, possessed the cpDNA of Haplotype D (1-4, 12, 13, 15, and 16). In Korea, the three populations from the central peninsula exhibited an endemic haplotype (type G) (44-46). Haplotype E showed a discontinuous distribution pattern; it was found on Sakhalin and in central and western Honshu (6, 9, 28, 31, 32, 34, and 39). Haplotypes B and I were each observed in a single population (21 and 41, respectively).

Pedicularis yezoensis, *P. schistostegia* and *P. chamissonis* were distinguished from Haplotype A of *P. resupinata* (sequence in plants from Mt. Hyonosen (38)) by 10 nucleotide substitutions and six indels, nine site changes and eight gaps, and 25 nucleotide substitutions and five indels, respectively.

Phylogenetic analysis among cpDNA haplotypes

To clarify the phylogenetic relationship among the nine-cpDNA haplotypes of *Pedicularis resupinata*, I further sequenced two non-coding regions of cpDNA representative of each haplotype of this species and three outgroup species (Table 1). The length of the intergenic regions between *trnT* (UGU) and *trnL* (UAA) 5' exon varied from 713-715 bp, and the region included 10 bp of the coding region of *trnT*. In this region, 57 nucleotide substitutions and five indels were found among all accessions (12 sequences), and 17 site changes and three indels were detected within the cpDNA haplotypes of *P. resupinata*. The length of the intergenic regions

TABLE 2. The chloroplast DNA haplotypes of *Pedicularis resupinata*.

	trnL intron								*trnL-F* spacer						
Position	1	1	1	2	3	6	6	6	7	7	8	8	8	9	9
	1	3	6	4	9	5	8	9	0	5	0	0	4	0	1
Haplotype	6	9	7	1	7	6	5	5	6	8	0	9	2	7	1
A	------	A	T	----	C	T	A	T	--------	T	T	G	GT	T	-
B	******	*	*	****	*	*	*	*	********	G	*	*	**	*	*
C	******	*	C	****	*	*	*	*	********	*	*	*	**	*	*
D	******	*	*	****	*	*	*	*	********	*	*	A	**	*	*
E	******	*	*	****	*	*	G	*	********	*	C	*	**	*	*
F	******	*	*	****	*	*	*	G	********	*	C	*	**	*	*
G	******	*	*	****	*	C	*	*	********	*	*	*	A*	G	*
H	AACAAA	G	C	AAAC	T	*	*	*	********	*	*	A	AG	G	T
I	AACAAA	G	C	****	T	*	*	*	TCTTATCA	*	*	A	AG	G	T

A dot denotes site changes or indels identical to the characteristics of Haplotype A.

between *atpB* and *rbcL* varied from 732-739 bp, and 15 site changes and seven gaps were detected among all accessions, and five site changes and three indels were detected in the intraspecific level of *P. resupinata*. The total length of the combined data set after multiple alignments of the three regions was 2473 bp, while 117 site changes and 30 gaps were inferred among all accessions; 34 site changes and 14 indels were detected at the intraspecific level. The DNA divergence values (uncorrected *p*-distances, Kumar *et al.* 1993), excluding indels among the cpDNA haplotypes, ranged between 0 and 1.11%. In the MP analysis, two of the five gaps in the *trnT-L* region and four of the eight indels of the *atpB-rbcL* region were not used (data not shown).

Wagner parsimony analysis of nine cpDNA haplotypes of *Pedicularis resupinata* based on the characteristics of both site changes and indels resulted in 105 parsimonious trees using the branch and bound search option. The trees required 153 steps; Consistency Index including uninformative characters (CI) = 0.9281, Retention Index (RI) = 0.8981. The strict consensus tree (MP tree) of the 105 trees is shown in Fig. 3. The MP tree displayed the following phylogenetic relationships. First, the cpDNA haplotypes of *P. resupinata* formed a clade with 74% bootstrap probability against the outgroups. Two major clades were revealed within the haplotypes of *P. resupinata* (Clades I and II). Clade II comprised cpDNA haplotypes H and I with a bootstrap value of 100%. Clade I included all remaining haplotypes (A-G) with a 100% bootstrap probability. Within this clade, Haplotype G was sister to the other haplotypes (Clade III; A-F) with a bootstrap value of 100%. The phylogenetic relationships within Clade III were unresolved polytomies, but haplotypes E and F formed a clade (Clade IV) with a bootstrap value of 88%.

The NJ tree based on the Kimura two-parameter model is shown in Fig. 4. The topology of the NJ tree was almost the same as that of the MP tree. The monophyly of the haplotypes of *P. resupinata*, however, was weakly supported (38%).

Discussion

Two major clades (Clades I and II) were revealed in the cpDNA haplotypes of *Pedicularis resupinata* with high bootstrap probability (Figs. 3 and 4), suggesting that the haplotypes of each clade diverged from a common ancestral genome. Clade I included seven haplotypes, and Clade II comprised only two haplotypes (types H and I). The

haplotypes of the latter clade were distributed only in central Honshu and Shikoku, Japan (Fig. 1) in populations at relatively high altitudes (Table 1). The remaining haplotypes, belonging to Clade I, were distributed widely in the Japanese islands (excluding Shikoku), the Korean peninsula, Sakhalin, and the Kuril islands. In short, the haplotypes of Clade II have a restricted distribution and are endemic haplotypes in Japan. These findings may indicate that the cpDNA haplotypes of Clade II originated within the Japanese islands, where they remain as relics in central Honshu and on Shikoku.

In Japanese alpine plants, the endemic clade distributed in central Honshu was inferred from the phylogeographical analysis using cpDNA; *Pedicularis chamissonis* Steven (Fujii *et al.* 1997), *Primula cuneifolia* Ledeb. (Fujii *et al.* 1995, 1999). Both species occur in the subalpine-alpine area of Japan, and are distributed in the North Pacific coastal area from the Japanese archipelago northeastwards to southwest Alaska. These studies showed that one clade was distributed in central Honshu and the other clades were present in northern Honshu and northward. They assumed that the clade distributed in central Honshu entered the Japanese islands earlier than did the northern clades, and considered that central Honshu played the role of a refugia for the species in the interglacial periods of the Pleistocene epoch. In the present analyses, clade II of *P. resupinata* is endemic to central Honshu and Shikoku (Fig. 1), although the distribution pattern of the clade differs from those of *P. chamissonis* and *Pr. cuneifolia*. Clade II of *P. resupinata* may have arisen during the migration processes that took place during changes in the climatic during the Pleistocene.

Two populations from central Honshu (nos. 26 and 33), identified as *Pedicularis resupinata* var. *caespitosa*, possessed the cpDNA profile of Clade II (type H). This variety, which occurs in the subalpine area of central to northern Honshu, is distinguished by having capitate inflorescences (Koidzumi 1925, Kitamura 1958, Yamazaki 1981, 1993, Shimizu 1982). Although plants in other populations with the cpDNA pattern of Clade II (23, 36, 37, 40, and 41) were identified as var. *oppositifolia*, var. *caespitosa* was observed only in Clade II. Variety *caespitosa* may have differentiated from the common ancestor of Clade II.

In Clade I, the cpDNA haplotype G was sister to haplotypes A-F (Clade III) with high bootstrap probability (Figs. 3 and 4). Haplotype G was observed only in central Korea; the northern and southern Korean populations showed the cpDNA of type A in Clade III (Fig. 1). This evidence suggests that Haplotype G diverged early from the common ancestor of Clade I and remained in the Sorak mountain area of the Korean Peninsula. In my field observations in 2000 in the Sorak mountains, I saw only plants with a white corolla. They were described as *Pedicularis resupinata* var. *resupinata* f. *albiflora* Y. N. Lee (Lee 1996). Most of the plants of *P. resupinata* have a pinkish red or reddish purple corolla. I did not see any pinkish flowers during my visit, which may indicate that morphological differentiation, at least in corolla color, has occurred in the populations of the Sorak Mountains in Korea.

The chloroplast genomes of haplotypes A-F of Clade III had the most derivative position in the trees, but the genetic differentiations among the haplotypes were relatively small (Figs. 3 and 4). The distribution area of the clade was wide, especially that of Haplotype A (Fig. 1). These findings suggest that the genomes of Clade III diverged and expanded their distribution area more recently than those of types G-I in northeastern Asia. Furthermore, they may have differentiated in central Honshu (types B and F), northern Honshu (type C), and in the Kuril islands and Hokkaido (type D).

Haplotypes E and F of Clade III form their own clade (Clade IV). Most plants of Clade IV are in central Honshu, but some are also on Sakhalin

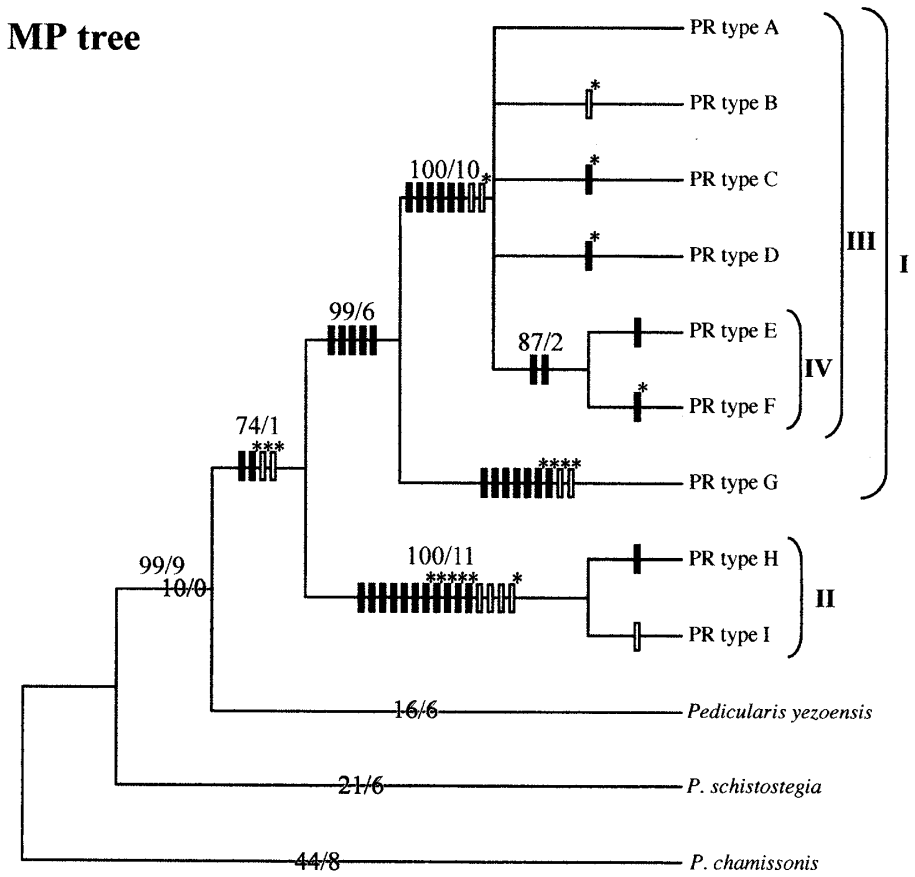

FIG. 3. Strict consensus of 105 most parsimonious trees for *Pedicularis resupinata* cpDNA haplotypes derived from *trnT-trnL*, *trnL-F* and *atpB-rbcL* non-coding regions. Numbers above branches are bootstrap values, in percentages, based on 1,000 replicates, and decay indexes, respectively. Solid and open bars represent site changes and indels, respectively. Bar with asterisk indicates homoplastic characters. Numbers on branches are numbers of site changes and indels, respectively.

(Fig. 1). Individuals in two populations from the Tokai district (nos. 34 and 35) with cpDNA of clade IV were identified as *Pedicularis resupinata* var. *microphylla*. Variety *microphylla* occurs mainly in the Tokai district of Honshu and is among the "Tokai hilly land elements," which are relic taxa in the region surrounding Ise Bay (Ueda 1989, 1994). It is believed that taxa of this association have adapted to the unique, small, peat-free swamps and marshes in the region. In this analysis, var. *microphylla* was recognized only in Clade IV, suggesting that plants of var. *microphylla* differentiated from a part of clade.

According to Yamazaki's (1981) hypothesis on the evolutionary history of *Pedicularis resupinata*, var. *microphylla* is derived from var. *oppositifolia*, and vars. *resupinata* and *caespitosa* entered Japan from the north. If his hypothesis is correct, it is expected that individuals of vars. *microphylla* and *oppositifolia* would form a clade, and vars. *resupinata* and *caespitosa* would constitute a sister group. In my cpDNA analysis, however, plants identified as var. *oppositifolia* occurred in all of the clades (I-IV), while those identified as vars. *resupinata* and *caespitosa* were placed in different clades (I and II) (Figs. 3 and 4). Yamazaki's hypothesis was therefore not supported by cpDNA analysis. CpDNA capture through introgressive hybridization or lineage sorting, which has been reported in many plant taxa by Wendel & Doyle (1998) could explain the discrepancy. To verify the possibility, we need to examine the phylogenetic

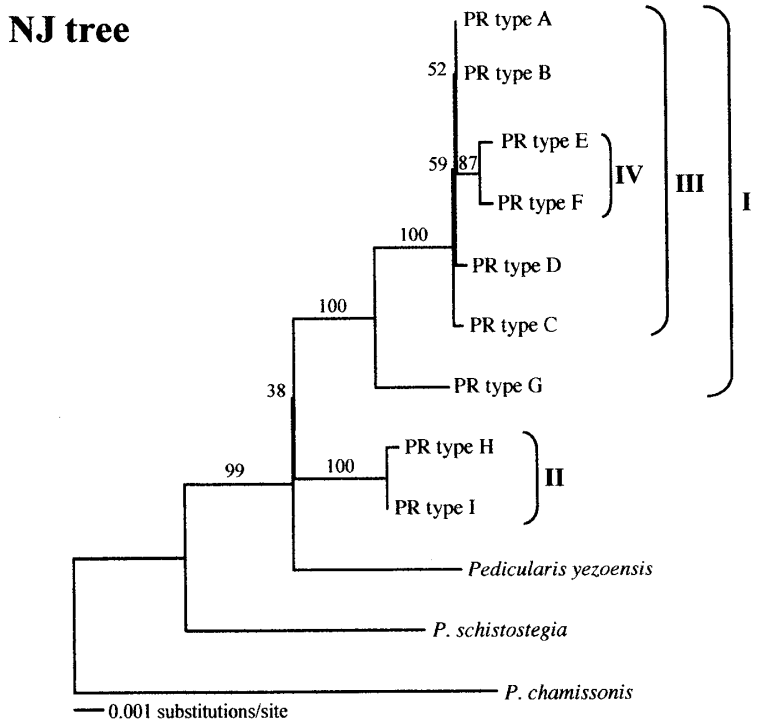

FIG. 4. Neighbor-joining tree based on Kimura's (1980) two-parameter model for *Pedicularis resupinata* cpDNA haplotypes was derived from *trnT-trnL*, *trnL-F* and *atpB-rbcL* non-coding regions. Numbers above branches are bootstrap values, in percentages, based on 1,000 replicates.

relationships among the populations of the group using the nuclear maker.

In 1993 Yamazaki proposed a new infraspecific taxonomic system for the *Pedicularis resupinata* group in which he recognized three subspecies: *resupinata*, *oppositifolia* (Miq.) Yamazaki, and *teucriifolia* (Bieb. ex Steven) Yamazaki. Within subsp. *oppositifolia* he recognized var. *oppositifolia* and var. *microphylla* (erroneously as 'mikawana'). Within subsp. *teucriifolia* he included var. *teucriifolia* and var. *caespitosa*. In contrast to his 1981 treatment, Yamazaki (1993) recognized the plants from Hokkaido and the southern Kuril islands as var. *teucriifolia*. In this analysis of the cpDNA, most plants from Hokkaido and the Kuril islands exhibited the cpDNA of haplotype D (Fig. 1), thereby supporting Yamazaki's 1993 treatment. In the phylogenetic analyses, however, haplotype D is included in Clade III, and haplotype H of var. *caespitosa* in Clade II (Figs. 3 and 4). Although Yamazaki (1993) did not refer to the evolutionary history of the infraspecific taxa of *P. resupinata*, his new system is not supported by our cpDNA analysis.

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